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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1666635> since 2019-04-05T15:52:00Z

*Published version:*

DOI:10.1158/1078-0432.CCR-17-1914

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## **BRAF and MEK inhibitors increase PD1-positive melanoma cells leading to a potential lymphocyte-independent synergism with anti-PD1 antibody**

Martina Sanlorenzo<sup>1,2,3</sup>, Igor Vujic<sup>4,5</sup>, Arianna Floris<sup>1</sup>, Mauro Novelli<sup>2</sup>, Loretta Gammaitoni<sup>6</sup>, Lidia Giraudo<sup>6</sup>, Marco Macagno<sup>1</sup>, Valeria Leuci<sup>1</sup>, Ramona Rotolo<sup>1</sup>, Chiara Donini<sup>1</sup>, Marco Basiricò<sup>6</sup>, Pietro Quaglino<sup>2</sup>, Maria Teresa Fierro<sup>2</sup>, Silvia Giordano<sup>1</sup>, Maria Sibilia<sup>3</sup>, Fabrizio Carnevale-Schianca<sup>6</sup>, Massimo Aglietta<sup>1,6</sup>, Dario Sangiolo<sup>1,6</sup>

<sup>1</sup>Department of Oncology, University of Turin – 10124, Turin, Italy

<sup>2</sup>Department of Medical Sciences, Section of Dermatology, University of Turin –10126, Turin, Italy

<sup>3</sup>Institute of Cancer Research, Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna – 1090, Vienna, Austria.

<sup>4</sup>The Rudolfstiftung Hospital, Department of Dermatology – 1030, Vienna, Austria

<sup>5</sup>School of Medicine, Sigmund Freud University Vienna – 1020, Vienna, Austria

<sup>6</sup>Division of Medical Oncology - Experimental Cell Therapy, Candiolo Cancer Institute, FPO - IRCCS – 10060, Candiolo (Turin), Italy

### **Correspondence should be addressed to:**

Dr. Martina Sanlorenzo

Department of Oncology, University of Turin, Candiolo Cancer Institute, FPO - IRCCS – Km 3,95, SP142, 10060, Candiolo (Turin), Italy (ISO 3166-2:IT), Phone: +390119933521, Fax: +390119933522, e-mail: [martina.sanlorenzo@ircc.it](mailto:martina.sanlorenzo@ircc.it)

**Running title:** BRAF and MEK inhibitors increase PD1-positive melanoma cells

The authors declare no potential conflicts of interest.

**Statement of translational relevance:**

BRAF and MEK inhibitors lead to increased rates of melanoma cells '*ectopically*' expressing PD1, supporting a lymphocyte-independent antitumor effect of anti-PD1 antibody. This provides further rationale for BRAF and MEK inhibitors/anti-PD1 antibody combination therapies in metastatic melanoma patients.

## ABSTRACT

**Purpose:** BRAF and MEK inhibitors (BRAF/MEKi) favor melanoma-infiltrating lymphocytes, providing the rationale for current combinatorial trials with anti-PD1 antibody. A portion of melanoma cells may express PD1, and anti-PD1 antibody could have a direct anti-tumor effect. Here, we explore if BRAF/MEKi modulate rates of PD1<sup>+</sup> melanoma cells, supporting an additional – lymphocyte-independent – basis for their therapeutic combination with anti-PD1 antibody.

**Experimental design:** With data mining and flow cytometry, we assessed PD1, PDL1/2 expression on melanoma cell lines (CCLE, N=61; validation cell lines, N=7) and melanoma tumors (TCGA, N=214). We explored *in-vitro* how BRAF/MEKi affect rates of PD1<sup>+</sup>, PDL1/2<sup>+</sup> melanoma cells, and characterized the proliferative and putative stemness features of PD1<sup>+</sup> melanoma cells. We tested the functional lymphocyte-independent effect of anti-PD1 antibody alone and in combination with BRAF/MEKi *in-vitro* and in an *in-vivo* immunodeficient murine model.

**Results:** PD1 is consistently expressed on a small subset of melanoma cells, but PD1<sup>+</sup> cells increase to relevant rates during BRAF/MEKi treatment (7.3% [5.6-14.2] vs 1.5% [0.7-3.2], p=0.0156; N=7), together with PDL2<sup>+</sup> melanoma cells (8.5% [0.0-63.0] vs 1.5% [0.2-43.3], p=0.0312; N=7). PD1<sup>+</sup> cells proliferate less than PD1<sup>-</sup> cells (avg. 65% less; t=7 days), and are preferentially endowed with stemness features. *In-vivo*, the direct anti-melanoma activity of PD1-blockage as monotherapy was negligible, but its association with BRAF/MEKi significantly delayed the development of drug resistance and tumor relapse.

**Conclusions:** BRAF/MEKi increase the rates of PD1<sup>+</sup> melanoma cells that may sustain tumor relapse, providing a lymphocyte-independent rational to explore combinatory strategies with anti-PD1 antibody.

## Introduction

Metastatic melanoma is still deadly, despite novel immunomodulatory and protein kinase inhibitor therapies. In preclinical studies, combinations of anti-PD1 antibody and target therapy with BRAF/MEK inhibitors (BRAF/MEKi) had synergistic effects, explained by an increased number and activity of tumor-infiltrating lymphocytes (1,2). This increase of tumor-infiltrating lymphocytes following BRAF/MEKi treatment is well documented (3,4), but the tumors may evade the immune system through expression of programmed death-receptor-ligand 1 (PDL1) and 2 (PDL2). These ligands bind and activate the programmed death-receptor 1 (PD1) on T-lymphocytes and suppress the antitumor response (5), while its blockage - by anti-PD1 antibody - restores the antitumor effect. However, it was suggested that PD1 is '*ectopically*' expressed also on melanoma cells, and that its activation could promote tumor growth (6,7). The biological relevance of these findings is still not clear, but PD1<sup>+</sup> melanoma cell subsets were found to preferentially express tumor-initiating determinants (6,7). Such putative cancer stem cells could contribute to the development of drug resistance and tumor relapse (8–10), which is a main issue for patients treated with BRAF/MEKi (11–13). In fact, after an initial rapid anti-tumor response, most patients experience disease progression despite ongoing treatment (11–13). Therefore, there is the need to elucidate the relevance of PD1<sup>+</sup> melanoma cells during BRAF/MEKi treatment, and to define therapeutic approaches, which could contrast the development of resistance to target therapies.

Here, we evaluate the '*ectopic*' melanoma-intrinsic PD1 expression and show that PD1<sup>+</sup> and PDL2<sup>+</sup> melanoma cells increase during BRAF/MEKi treatment, sensitizing tumor cells to direct anti-PD1 antibody effects, thus delaying the development of resistance to target therapy.

## Material and Methods

Cancer Cell Line Encyclopedia (CCLE): We extracted from the CCLE the mRNA expression values of the 61 available melanoma cell lines (<http://www.broadinstitute.org/ccle>, access September 2015).

The Cancer Genome Atlas (TCGA): We downloaded from the TCGA portal the clinical data (clinical data, pathology report) and the mRNA sequencing data (gene) of the 470 melanoma samples included (<https://tcga-data.nci.nih.gov/tcga>, access September 2015). The mRNA sequencing data were available for 469 samples. Genes with FPKM values >0.1 were considered expressed (14). We matched each mRNA sequencing file with the corresponding clinical and histological data to divide the melanomas in primary, regional metastases and distant metastases and to exclude from further analyses all the samples with histological evidence of immune infiltrate.

Cell lines: Melanoma cell lines SKMEL2, SKMEL5, SKMEL28 were obtained from NCI-Frederick Cancer/DCTD Tumor Repository in 2011, and A375 (ATCC® CRL1619™) from ATCC in 2013. Cell line identity was performed by the bank of origin using morphology, karyotyping, and PCR based approaches. Mycoplasma detection was performed after cell thawing by Universal Mycoplasma Detection Kit ATCC (ATCC-30-1012K) according to the manufacturer's instructions. Cell lines for experiments were obtained from the original cryopreserved golden stock and experiments performed immediately after and for no longer than 6 months, no further cell identification was performed. SKMEL2, SKMEL5 and SKMEL 28 were maintained in RPMI (Sigma Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL). Melanoma cell line A375 cell was maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) with the addition of 2mM Glutamine and 15% FBS (Gibco BRL). All the cells were propagated at 37°C under 5% CO<sub>2</sub>. Cells were passaged and harvested from flasks using Accutase solution (Gibco BRL).

Patients derived samples: Patient-derived melanoma cell cultures (mMel2, mMel3, mMel7, mMel11) were generated from surgical biopsies of metastatic/locally advanced melanoma, before any systemic treatment (December 2010 – June 2012). All patients provided consent under institutional review board approved protocols. Technical procedures and melanoma cell cultures were previously described (10,15). Mycoplasma detection was performed by Mycoplasma PCR Detection Kit (Applied Biological Materials Inc., MICROTECH s.r.l. Napoli – Italy) according to the manufacturer's instructions. The test was done after cell thawing/just before the experiment execution. All the experiments were performed on patient-derived cell culture of not more than 24 week-old.

Generation of hOct4.eGFP transduced cell lines: The previously described lentiviral vector (14) was transduced in melanoma primary cells resuspended in fresh KODMEM-F12 (Gibco BRL) with 10% FBS adding virus-conditioned medium at a dose of 400 ng P24/100,000 cells. The lentiviral vector pRRL.sin.PPT.hOct4.eGFP.Wpre (LV-Oct4.eGFP) was obtained as previously described (15). Briefly, the hOct4-eGFP cassette from phOct4.eGFP1 vector (16) (kind gift from Dr. Wei Cui, IRDB, Imperial College, London) was cloned into the transfer vector pRRL.sin.PPT.hPGK.eGFP.Wpre (17) (kindly provided by Dr. Elisa Vigna, IRCCS Candiolo/University of Turin, Italy) in place of the hPGK.eGFP cassette. After 16 hours, cells were washed twice and grown for a minimum of 10 days to reach steady state eGFP expression and to rule out pseudotransduction before flow cytometry analysis. Technical procedure including transduction controls were previously described (10).

Drugs: The BRAF inhibitor dabrafenib (GSK2118436) and the MEK inhibitor trametinib (GSK1120212) were purchased from Selleckchem. The anti-PD1 antibody is the *inVivo*MAB anti-human PD1(CD279), Clone: J110 from Bio X Cell. The isotype control antibody is the *inVivo*MAB mouse IgG1 isotype control, Clone: MOPC-21 from Bio X Cell. Drugs were used accordingly to previous reports (7,18,19).

Flow cytometry: Analyses of melanoma cells were performed using a CyanADP cytometer (Beckman Coulter s.r.l, Cassina De' Pecchi – Milan, Italy). The fluorochrome-conjugated monoclonal antibodies included anti-PDL1 PE (clone MIH1); anti PDL2 APC (clone MIH18); anti PD1 APC (clone MIH4) from BD Biosciences. The negative staining threshold was established by the addition of an isotype-matched control tube.

In vitro proliferation assay and CFSE staining: To evaluate the proliferation rate, cells had been labeled with 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), for which fluorescence intensity decreased by half at each cell division per kit protocol (Sigma Aldrich). Briefly, the CFSE dye solution was prepared accordingly to the number of cells to stain and added to the previously washed cell pellet. After a first 15-minutes incubation at 37°C, cells were washed once with culture medium added with 10% heat-inactivated serum and incubated in culture medium added with 10% heat-inactivated serum for 30 minutes at 37°C. An aliquot of these labeled and counted cells was read on a Flow Cytometry Cyan (Cyan ADP, Beckman Coulter s.r.l.) and analyzed using Summit Software (Daki Cytomation, Heverlee, Belgium) to set the baseline fluorescence level. The remaining cells were seeded in culture under experimental conditions. After 4, and 7 days the reduction in fluorescence was quantified by flow cytometry. In case of drug treatments, treated cells were compared with untreated cells which were also labeled with the same dye.

In vitro cell viability assay: To test the number of viable, metabolically active cells after treatment with BRAF/MEKi alone or in combination with anti-PD1 antibody we used a method based on the quantitation of ATP present (CellTiter-Glo Luminescent Cell Viability Assay, Promega Italia s.r.l) according to the manufacturer's protocol.

In vivo: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were purchased from Charles River Laboratories Italia s.r.l. (Calco - Lecco, Italy). Mice were injected subcutaneously with  $1,5 \times 10^6$  A375 melanoma cells and randomly assigned to treatment groups. Treatments were started when tumors



became palpable and continued for 40 days. Dabrafenib and trametinib were administered by oral gavage 5 consecutive days a week at a dose of respectively 600µg and 4µg. Anti-PD1 antibody and respective isotype control mAb were injected intraperitoneally (200µg per injection) three times a week. Mice were sacrificed when the main tumor diameter reached 2 cm or massive ulceration occurred. All procedures were performed accordingly Institutional Review Board–approved protocols.

Statistical analyses: The statistical analyses were performed using Stata 12.0 statistical software (Stata, College Station, TX), and Prism7 (GraphPad Software, Inc). All variables were tested for normal distribution with the Shapiro- Wilk test, and none of them was found normally distributed. Comparisons between two independent non-normally distributed groups were performed using the nonparametric Wilcoxon rank-sum test. Comparisons between matched groups were performed with Wilcoxon signed rank test. Correlations between variables were tested with the Spearman’s rank correlation test. Differences in tumor volumes were statistically assessed using repeating measurements two-way ANOVA followed by Sidak correction and with two-tails. P values less than 0.05 were considered statistically significant.

## Results

### *Melanoma cells express low but consistent levels of PD1*

To investigate PD1 expression on melanoma cells, we analyzed two datasets: the Cancer Cell Line Encyclopedia (CCLE), and The Cancer Genome Atlas (TCGA).

All 61 CCLE melanoma cell lines expressed PD1 with mRNA values comparable to those of PDL1 and PDL2 (Affymetrix mRNA values: PD1 4.20 [3.81-4.65], PDL1 4.63 [3.73-7.84], and PDL2 3.73 [3.97-8.50]) (Fig. 1A). Average melanoma PD1 values were about 40% of those of the established melanoma antigen s100B (Affymetrix mRNA values: 10.39 [3.28-13.80]), used as an internal

control putting the mRNA values into perspective (Fig. 1B), and around 95% of the average PD1 expression found in 18 T-cell neoplasia cell lines included in the CCLE (Affymetrix mRNA values: 4.39 [3.98-5.34]) (Fig. 1C).

From the 470 TCGA patient derived melanomas, we matched gene expression data with corresponding histological reports and we excluded all the samples with histological evidence of immune infiltrate, as those would interfere with the assessment of melanoma-intrinsic PD1 expression (Fig. 1D). PD1 was expressed in 99.5% of the samples, with a median expression comparable to PDL1 and PDL2 (Fig. 1E). We did not find significant differences when we compared samples with (N=100) and without (N=114) stromal cells (median FPKM values 32.5 [0-1282.8], and 64.7 [0.4-1461.3] respectively,  $p=0.0970$ ). Furthermore, we observed positive correlations between PD1 and PDL1 ( $r=0.66$ ,  $p<0.0001$ ), PD1 and PDL2 ( $r=0.80$ ,  $p<0.0001$ ), and PDL1 and PDL2 ( $r=0.79$ ,  $p<0.0001$ ) (Fig. S1).

#### *BRAF/MEKi treatment increases rates of PD1<sup>+</sup> and PDL2<sup>+</sup> melanoma cells*

Next, we assessed by flow-cytometry PD1 protein levels in BRAF<sup>V600</sup> and NRAS<sup>Q61</sup> mutant melanoma cell lines. PD1 was expressed in all these cell lines, but the median percentage of PD1<sup>+</sup> viable cells was only 1.2% [0.7-4.3] (N=7). Median expression levels of PDL1 and PDL2 were 1.5% [0.1-53.1], and 1.1% [0.2-51.7] (N=7).

BRAF and MEK inhibitor combinations are currently widely used in the treatment of BRAF mutant melanoma patients (20), and MEK inhibitors showed efficacy in the treatment of NRAS mutant melanoma (21,22). Therefore, we tested if BRAF/MEKi affect rates of PD1<sup>+</sup> melanoma cells, as this could have implications for combination therapies. We treated melanoma cells with the BRAF inhibitor dabrafenib, the MEK inhibitor trametinib and their combination. The chemotherapeutic agent fotemustine served as a control, as it efficiently inhibits tumor growth, but does not interfere with the MAPK pathway (23). DAPI staining was used to assess treatment efficacy and select viable

cells (Fig. 2A). During BRAF/MEKi treatment the rates of PD1<sup>+</sup> melanoma cells increased in all tested melanoma cell lines (median values: 7.3% [5.6-14.2] vs 1.5% [0.7-3.2],  $p=0.0156$ ;  $N=7$ ) (Fig. 2B, Fig. S2A). While the rates of PDL1<sup>+</sup> melanoma cells did not change (1.3% [0.0-62.3] vs 1.3% [0.0-53.1],  $p>0.05$ ;  $N=7$ ) (Fig. 2C, Fig.S2B), we found a significant increase of PDL2<sup>+</sup> melanoma cells during BRAF/MEKi treatment (8.5% [0.0-63.0] vs 1.5% [0.2-43.3],  $p=0.0312$ ;  $N=7$ ) (Fig. 2D, Fig.S2C).

In BRAF<sup>V600</sup> mutant cells, the combination of BRAF and MEK inhibitors led to the highest percentage of PD1<sup>+</sup> cells, and showed the greatest anti-tumor effect, whilst in the NRAS<sup>Q61</sup> mutant cell line this was observed with MEK inhibitor alone (Fig. S3). Fotemustine efficiently killed tumor cells, but did not significantly change levels of PD1<sup>+</sup> cells (Fig. 2E).

#### *Rates of PD1<sup>+</sup> melanoma cells increase in a time- and drug- dependent manner during BRAF/MEKi treatment*

We explored if the percentage of PD1<sup>+</sup> melanoma cells was influenced by time or drug exposure. Increasing the time of treatment with BRAF/MEKi, we observed a progressive increase of the percentage of PD1<sup>+</sup> cells. After 8 days of treatment, viable PD1<sup>+</sup> melanoma cells increased to 31.8% on average [15.0-50.3%] ( $N=3$ ) (Fig. 2F, 2G). Following BRAF/MEKi withdrawal, the rate of PD1<sup>+</sup> melanoma cells returned back to the low original value (Fig. 2H, 2I) ( $N=3$ ).

#### *PD1<sup>+</sup> melanoma cells are more quiescent and present stemness features*

Since during BRAF/MEKi treatment PD1<sup>+</sup> cells reach significant percentages among the viable tumor cell population, we compared their proliferative capabilities with PD1<sup>-</sup> cells. We used a carboxyfluorescein succinimidyl ester (CFSE) dye-based assay, where the dye decrease corresponds to higher mitotic activity and faster proliferation rate. Treatment with BRAF/MEKi decreased the overall proliferation (Fig. 3A) with PD1<sup>+</sup> cells proliferating less than the PD1<sup>-</sup> counterparts; on average 16.1% less after 96 hours ( $N=3$ ), and 65% less after 7 days ( $N=2$ ) (Fig. 3B, Fig. S4).

PD1 was reported to be preferentially expressed on putative melanoma cancer stem cells (6). To test this, we used a lentiviral vector carrying eGFP under the transcriptional control of the Oct4 stemness gene promoter (LV-Oct4.eGFP) (Fig. 3C)(15). This system visualizes putative cancer stem cells as eGFP<sup>+</sup>, based on their selective ability to activate the Oct4 promoter. In three LV-Oct4.eGFP transduced patient-derived cell lines (mMel2-Oct4, mMel3-Oct4, mMel7-Oct4), BRAF/MEKi led to overall eGFP<sup>+</sup> cell enrichment (on average 2.4-fold), suggesting a lower sensitivity of eGFP<sup>+</sup> melanoma cells to these drugs (Fig. 3D). Moreover, eGFP<sup>+</sup> putative cancer stem cells were enriched among PD1<sup>+</sup> cells compared to PD1<sup>-</sup> cells (on average 1.6-fold) (N=3) (Fig. 3E).

#### *Anti-PD1 antibody prolongs the antitumor response to BRAF/MEKi*

Considering the hypothesis that PD1 activation could lead to melanoma proliferation (7), we tested if PD1-blockage could have a direct anti-tumor effect.

*In-vitro*, the sole use of anti-PD1 antibody did not affect cell viability (Fig. S5). When we combined anti-PD1 antibody with BRAF/MEKi we observed only a trend towards a better anti-tumor effect compared to BRAF/MEKi alone during short-term drug exposure (Fig. S6). To test the hypothesis that the subset of PD1<sup>+</sup> melanoma cells, which are preferentially endowed with stemness features, might contribute to the development of BRAF/MEKi resistance, we set up an *in-vivo* long-term experiment. We used non-obese diabetic/severe combined immunodeficient (NOD-SCID)/interleukin 2 receptor [IL2r] $\gamma^{\text{null}}$  (NSG) mice bearing palpable subcutaneous xenograft melanoma. The treatment with anti-PD1 antibody alone did not have any beneficial effect on tumor growth (N=6) (Fig. 4A). On the other side, when combined with BRAF/MEKi, anti-PD1 antibody (N=6) significantly prolonged the antitumor response and delayed melanoma relapse compared to controls treated only with BRAF/MEKi (N=6) (p=0.0006).

## Discussion

BRAF/MEK inhibitors (BRAF/MEKi) and anti-PD1 antibody combinations might be a therapeutic strategy for metastatic melanoma patients, and phase II and III clinical trials (NCT02910700, NCT02224781, NCT02130466, NCT02967692, NCT02858921) are currently recruiting. These trials are based on preclinical models which explain the synergism by positive BRAF/MEKi effects on T-cell recruitment, PDL1 up-regulation on tumor cells and consequent enhancement of anti-PD1 antibody antitumor effect (1,2). Our results point to a novel, lymphocyte-independent, mechanism of action: BRAF/MEKi treatment leads to higher rates of viable melanoma cells expressing PD1 and PDL2, and therefore it could sensitize the tumor to a direct inhibitory effect of anti-PD1 antibody. 'Ectopic' melanoma-intrinsic PD1 expression and its possible role in promoting tumor growth were proposed (7), but the biological relevance of these findings is still not clear. Such subsets were observed to have tumor-initiating properties; thus, they could contribute to the development of drug resistance.

We first chose an *in-vitro* platform to characterize melanoma-intrinsic PD1 expression in normal conditions and during BRAF/MEKi treatment. We confirmed that melanoma cells do express intrinsic PD1, but at very low rates. Such low percentage of PD1<sup>+</sup> cells is unlikely to account for large functional effects and indeed, treatment with anti-PD1 antibody alone affected neither tumor cell viability *in-vitro*, nor tumor growth in immunodeficient xenograft models.

However, upon treatment with BRAF/MEKi, percentages of PD1<sup>+</sup> cells increased to relevant numbers in all tested melanoma cell lines, likely capable to enhance tumor proliferation if activated.

Furthermore, we found that BRAF/MEKi also up-regulated the PD1-ligand PDL2 on melanoma, therefore a juxtacrine, pro-proliferative PD1-activation on melanoma is feasible and biologically plausible. This interaction would support melanoma proliferation and thus counteract desired anti-tumor effects of BRAF/MEKi.

The increased percentage of PD1<sup>+</sup> melanoma cells during treatment with BRAF/MEKi can be the result of a molecular modulation of PD1 protein expression, but also of a selective process of PD1<sup>+</sup> melanoma cells less sensitive to target therapy. The linear kinetics of PD1 expression during BRAF/MEKi treatment, and its rapid reversion upon drug withdrawal endorse the hypothesis of a dynamic modulation. On the other hand, our finding that the PD1<sup>+</sup> cell subsets are enriched with cells with stemness features supports the idea of a higher resistance of those cells to target therapy treatment. The description of the exact mechanism leading to the increase of PD1<sup>+</sup> melanoma cells during treatment with BRAF/MEKi is beyond the scope of this work. Instead, we focused our efforts to investigate the biological relevance of our findings.

*In-vitro*, the addition of anti-PD1 antibody to BRAF/MEKi, only slightly improved their anti-tumor effects, and only when used at higher dose (100 µg/ml) than previously described in melanoma (50 µg/ml) (7). Considering the negligible *in-vitro* effect, we did not further investigate possible off-target effects of anti-PD1 antibody. But the absence of any toxic or biological effect in melanoma cells treated exclusively with anti-PD1 antibody - where PD1 expression levels are very low (e.g. <1% in A375) – endorses the specific action of the drug. The *in-vitro* platform did not allow for long-term observations, and PD1<sup>+</sup> melanoma cells were more quiescent than their PD1<sup>-</sup> counterparts, potentially explaining the limited effect. On the other hand, the *in-vivo* long-term experiment demonstrated a significantly improved outcome in immunodeficient mice concomitantly treated with anti-PD1 antibody and BRAF/MEKi compared to mice treated with BRAF/MEKi alone. The NSG immunodeficient model was crucial to investigate the direct effect of PD1-blockage on melanoma in a lymphocyte-deprived context. For this *in-vivo* part of our study, we used the same dose of anti-PD1 antibody that was previously described with no evidences of off-target effect (7). Whilst mice treated with BRAF/MEKi alone, after an initial intense response, experienced a rapid tumor growth due to the development of drug resistance, mice treated with the combination of BRAF/MEKi and anti-PD1

antibody maintained the tumor response significantly longer. We found that cells with stemness features were enriched among PD1<sup>+</sup> cells. These putative cancer stem cells are endowed with the ability to initiate and promote tumor growth, and possibly contribute to BRAF/MEKi resistance (8–10). We speculate that their blockage with anti-PD1 antibody could contrast or delay the onset of resistance, and so explain the observed longer response of tumors treated with BRAF/MEKi and anti-PD1 antibody combinations.

Differently from previously published findings in the same mouse model (7), anti-PD1 antibody alone did not affect melanoma tumor growth in our hands. This difference can be due to the very low rates of PD1<sup>+</sup> melanoma cells and to a different treatment schedule. We started the treatment when mice had measurable tumors, whilst *Kleffel et al* (7) administered anti-PD1 antibody earlier, before the injection of melanoma cells. We believe that our model may be representative of realistic clinic scenarios, and suggest that the blockage of melanoma-intrinsic PD1 is indeed clinically relevant, but only when numbers of such PD1<sup>+</sup> cells increase, for example due to treatment with BRAF/MEKi.

In conclusion, our work describes that rates of PD1<sup>+</sup> melanoma cells increase during BRAF/MEKi treatment, and reveal how BRAF/MEKi could favor the direct action of anti-PD1 antibody on melanoma in absence of lymphocytes. Our findings give new clinical rationales to explore for concomitant administration of BRAF/MEK inhibitors and anti-PD1 antibody in metastatic melanoma patients.

## Acknowledgments:

Dr. M. Sanlorenzo is grateful to “L’Oréal Italia per le Donne e la Scienza” award for the support.

Dr. D. Sangiolo is supported by the FPRC ONLUS  $5 \times 1000$ , Ministero della Salute 2012;

“Associazione Italiana Ricerca sul Cancro” (AIRC) MFAG 2014 N.15731, IG20259; Ricerca

Finalizzata-Giovani Ricercatori Ministero della Salute (GR-2011-02349197), University of Torino

Fondo Ricerca Locale 2013. The results shown here are in part based upon data generated by the

TCGA Research Network: <http://cancergenome.nih.gov>.

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## Figure legends:

**Fig. 1. Melanoma cells express PD1** (A) Box plots of PD1, PDL1 and PDL2 Affymetrix mRNA expression values of 61 melanoma cell lines included in the Cancer Cell Line Encyclopedia (CCLE). (B) Bar graph of PD1 expression values compared to s100B in the same 61 melanoma cell lines. (C) Box plots representing median levels of PD1 in the 61 melanoma cell lines and in the 18 T-cell neoplasia cell lines included in CCLE (D) Flow chart of The Cancer Genome Atlas (TCGA) data analysis. Samples with histological immune infiltrate were excluded from further analyses. Box plots of PD1, PDL1 and PDL2 mRNA expression values of 214 melanomas without histological evidence of immune infiltrate.

**Fig. 2. BRAF/MEK inhibitors increase the rates of PD1<sup>+</sup> and PDL2<sup>+</sup> tumor cells in BRAF<sup>V600</sup> and NRAS<sup>Q61</sup> mutant melanomas** (A) Representative flow cytometry plots of a BRAF<sup>V600</sup> mutant melanoma cell line (A375) treated with dabrafenib [1μM], trametinib [5nM], the combination of dabrafenib + trametinib [1μM+5nM], and fotemustine [50μg/ml] for 96 hours. DAPI staining was used to identify viable cells. PD1, PDL1, PDL2 plots were performed considering only viable cells. Rates of (B) PD1, (C) PDL1, and (D) PDL2 positive melanoma cells untreated and after the treatment with BRAF/MEKi (dabrafenib+trametinib [1μM+5nM] in BRAF<sup>V600</sup> mutant cell lines and trametinib [5nM] in the NRAS<sup>Q61</sup> mutant cell line for 96 hours) (N=7), \* p<0.05. Median values in red. (E) Rates of PD1<sup>+</sup> cells in melanoma cells untreated and after fotemustine treatment [50μg/ml] for 96 hours (N=7). Median values in red. (F) Rates of PD1<sup>+</sup> melanoma cells at baseline, after 96h, and after 192h of BRAF/MEKi treatment (dabrafenib + trametinib [1μM+5nM]) (N=3). Average values and standard deviation. No significant differences were found. (G) Representative flow cytometry plots of A375 and mMel2 patient- derived melanoma cell lines treated with the combination of dabrafenib + trametinib [1μM+5nM] for prolonged time. (H) Rates of PD1<sup>+</sup> melanoma cells at baseline, after 96h,

and after 96h of BRAF/MEKi treatment + 96h of washout (dabrafenib + trametinib [1 $\mu$ M+5nM]) (N=3). Average values and standard deviation. No significant differences were found. **(I)**

Representative flow cytometry plots of A375 and mMel2 patient-derived melanoma cell lines treated with the combination of dabrafenib + trametinib [1 $\mu$ M+5nM] for 96h and after a 96h-time of wash-out. \*p<0.05, n.s = not significant.

**Fig. 3. PD1<sup>+</sup> melanoma cells have reduced proliferative potential and show stemness features.**

**(A)** Melanoma cell proliferation after 96h-treatment with BRAF/MEKi (dabrafenib+trametinib [1 $\mu$ M+5nM] in BRAF<sup>V600</sup> mutant cell lines and trametinib [5nM] in the NRAS<sup>Q61</sup> mutant cell line), compared to untreated controls measured with CFSE assay. N=3 (A375, SKMEL2, SKMEL5).

Average values in red. No significant differences were found. **(B)** Proliferation rates of PD1<sup>+</sup> melanoma cells compared to the PD1<sup>-</sup> counterparts after 96h (N=3) and 7 days (N=2) of

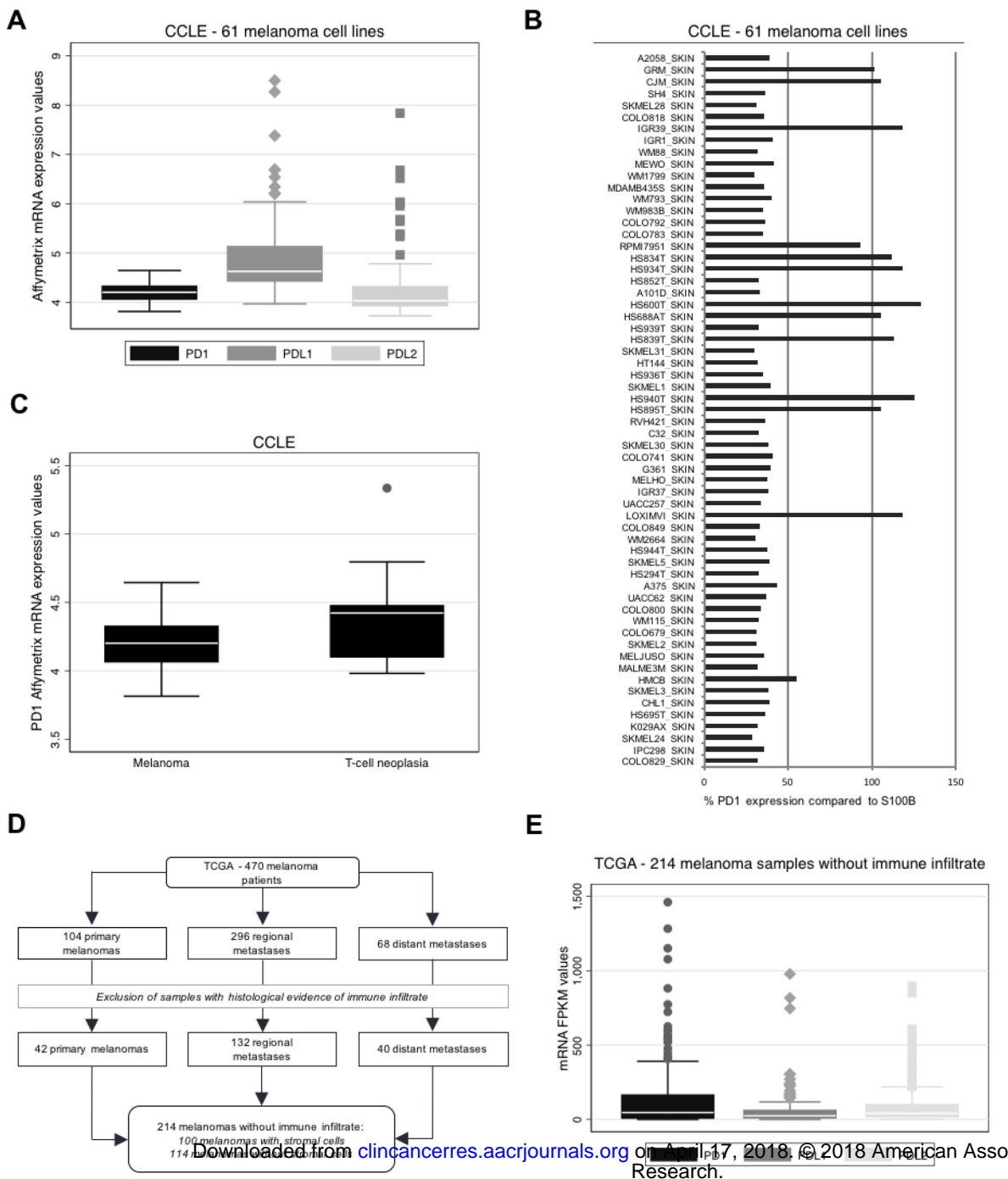
BRAF/MEKi treatment (dabrafenib + trametinib [1 $\mu$ M+5nM] in BRAF<sup>V600</sup> mutant cell lines and trametinib [5nM] in the NRAS<sup>Q61</sup> mutant cell line). No significant differences were found. **(C)**

Schematic representation of the lentiviral vector used to transduce melanoma cells; the eGFP expression is controlled by the promoter regulatory element of the Oct4 gene (LV-Oct4. eGFP). **(D)**

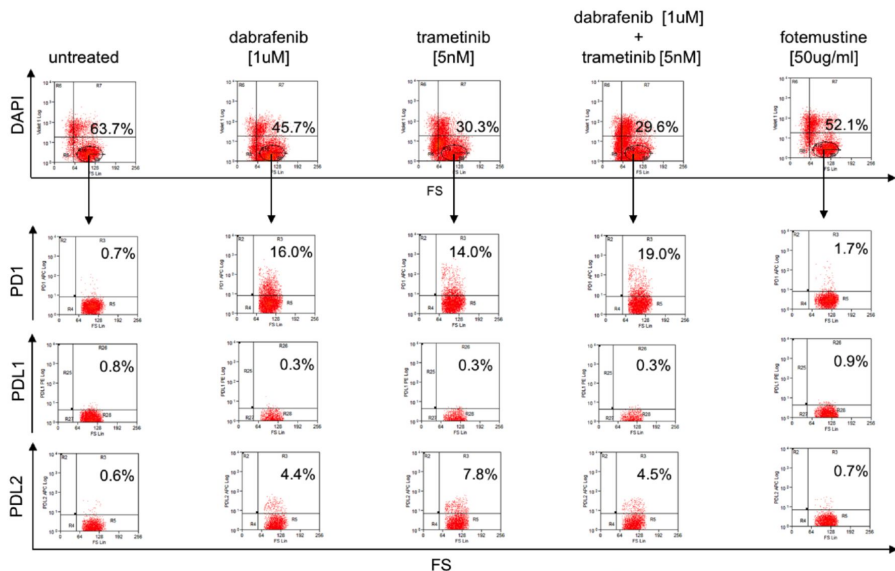
Percentage of eGFP<sup>+</sup> cells at baseline and after 96 hours of BRAF/MEKi treatment (dabrafenib + trametinib [1 $\mu$ M+5nM]), in patient-derived cell lines transduced with the LV-Oct4. eGFP vector

(N=3). Average values and standard deviation. No significant differences were found. **(E)** Percentage of eGFP<sup>+</sup>/PD1<sup>+</sup> cells compared to eGFP<sup>+</sup>/PD1<sup>-</sup> cells in patient-derived cell lines transduced with the LV-Oct4. eGFP vector (N=3). Average values and standard deviation. No significant differences were found.

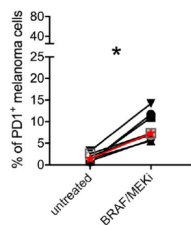
**Fig.4: Anti-PD1 antibody prolongs the antitumor response of BRAF/MEKi in immunodeficient mice.** (A) Kinetics (mean  $\pm$  SEM) of A375 xenograft growth in NSG mice treated with anti-PD1 antibody (anti-PD1 mAb) [200 $\mu$ g three times a week] (N=6) or isotype control antibody [200 $\mu$ g three times a week] (N=6). Treatment start is marked by the arrow. (B) Kinetics (mean  $\pm$  SEM) of A375 xenograft growth in NSG mice treated with BRAF/MEKi (dabrafenib + trametinib) [respectively 600 $\mu$ g and 4 $\mu$ g, five consecutive days a week] and anti-PD1 antibody (anti-PD1 mAb) [200 $\mu$ g three times a week] (N=6) or BRAF/MEKi (dabrafenib and trametinib) alone (N=6). Treatment start is marked by the arrow. Statistical analysis was carried out using two-way ANOVA followed by Sidak correction and with two-tails. \* $p < 0.05$ ; \*\*\* $p < 0.001$ )



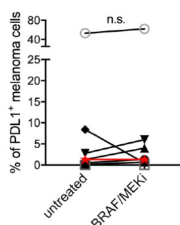
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A375 (BRAF<sup>V600E</sup>)

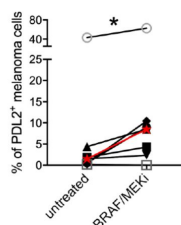
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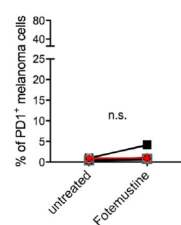
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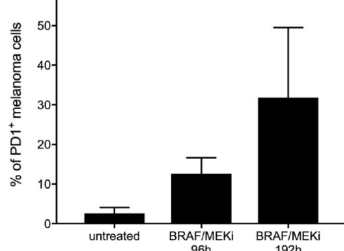


E

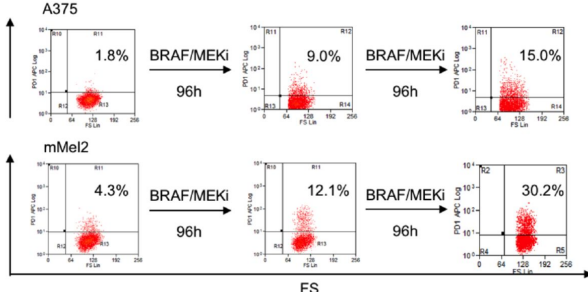


- ◆ A375 - BRAF<sup>V600E</sup>
- SKMEL5 - BRAF<sup>V600E</sup>
- ▲ SKMEL28 - BRAF<sup>V600E</sup>
- ▼ SKMEL2 - NRAS<sup>Q61R</sup>
- ◆ mMel11 - BRAF<sup>V600E</sup>
- ◇ mMel2 - BRAF<sup>V600K</sup>
- mMel7 - BRAF<sup>V600E</sup>

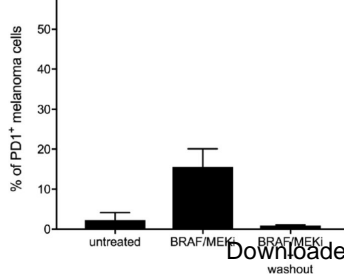
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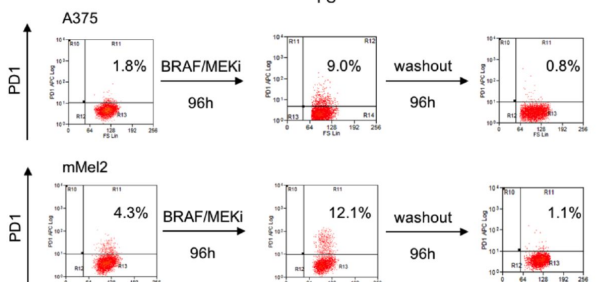
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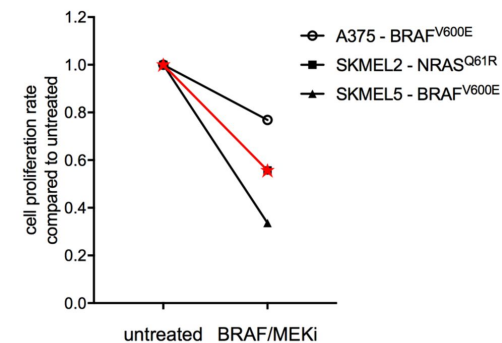
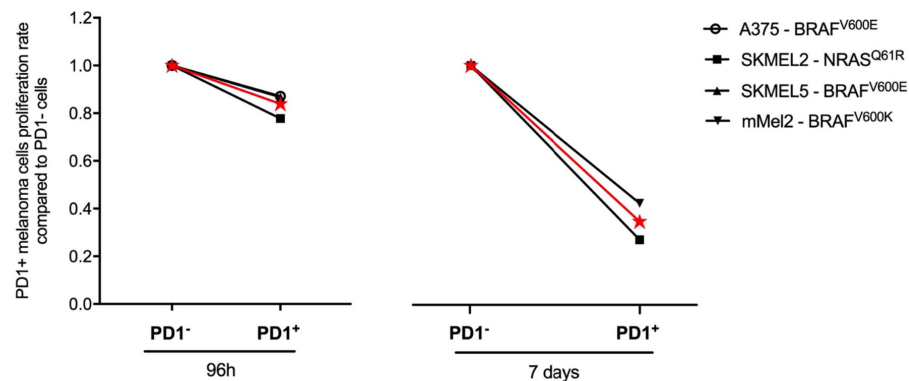
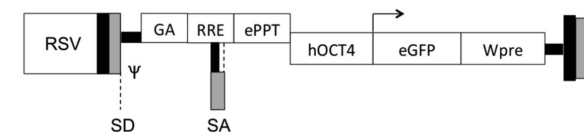
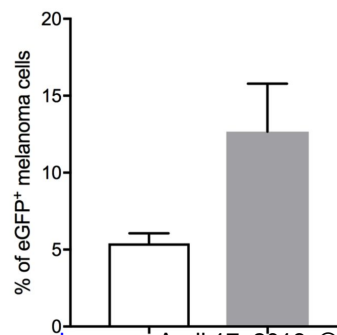
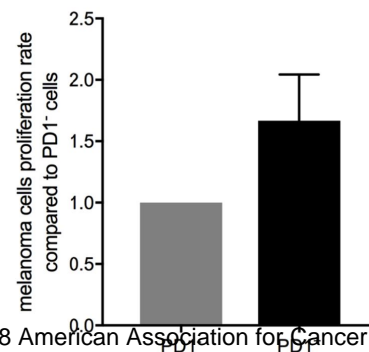
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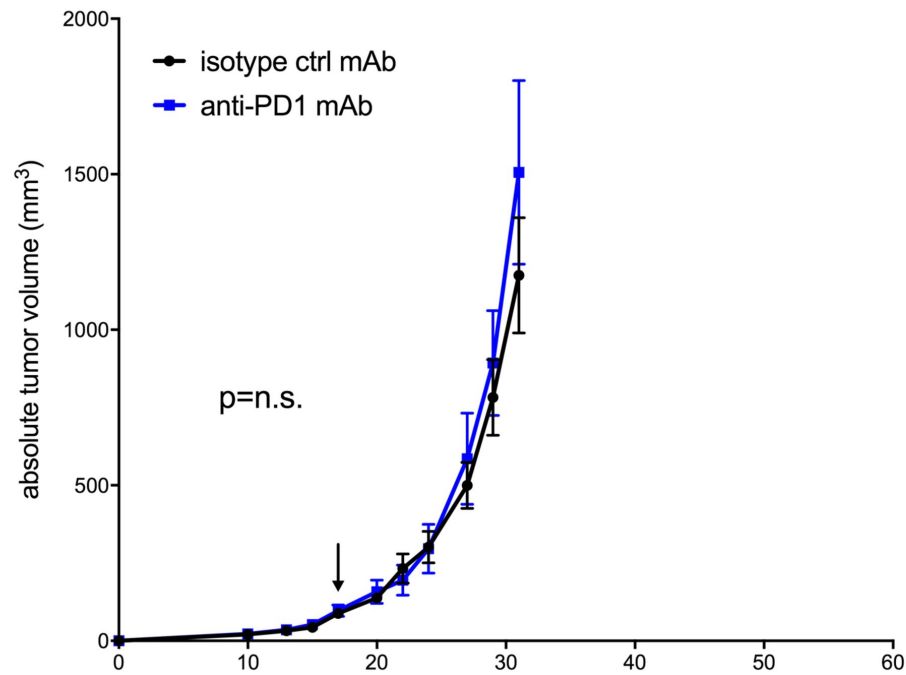
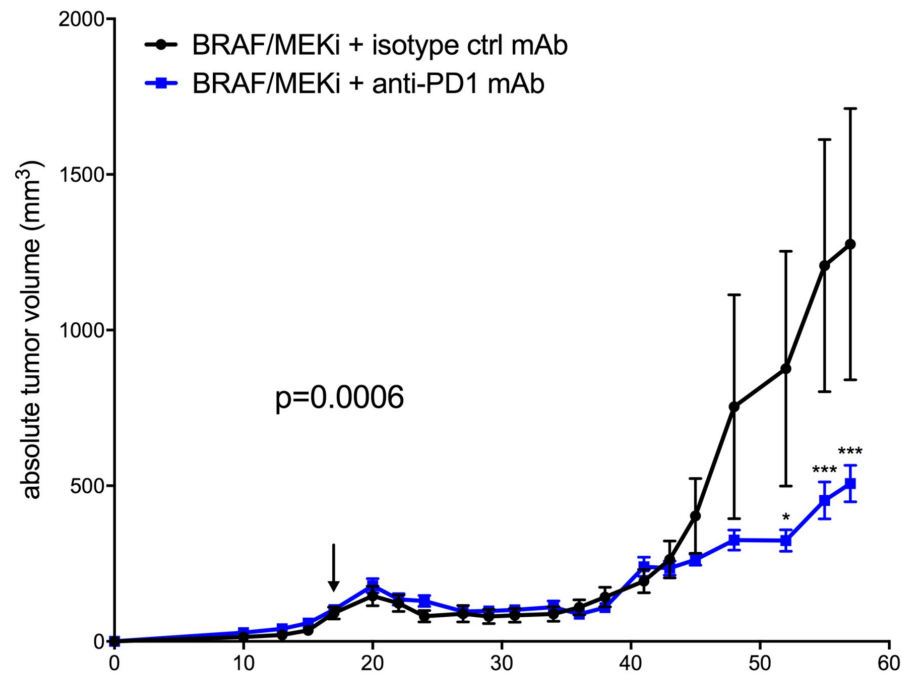


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**A****B**

# Clinical Cancer Research

## BRAF and MEK inhibitors increase PD1-positive melanoma cells leading to a potential lymphocyte-independent synergism with anti-PD1 antibody

Martina Sanlorenzo, Igor Vujic, Arianna Floris, et al.

*Clin Cancer Res* Published OnlineFirst April 12, 2018.

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